AD_____

Award Number: W81XWH-06-1-0557

TITLE: The Importance of Autophagy in Breast Cancer Development and Treatment

PRINCIPAL INVESTIGATOR: Jin-Ming Yang, Ph.D.

CONTRACTING ORGANIZATION: University of Medicine and Dentistry of New Jersey
New Brunswick, NJ 08903

REPORT DATE: June 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-06-2008	Annual	16 MAY 2007 - 15 MAY 2008

4. TITLE AND SUBTITLE

The Importance of Autophagy in Breast Cancer Development and Treatment

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0557

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jin-Ming Yang, Ph.D.

E-Mail: jyang@umdnj.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Medicine and Dentistry of New Jersey
New Brunswick, NJ 08903

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
In the current study, we tested the hypothesis that eEF-2 kinase plays a critical role in the ability of breast cancer cells to survive growth factor/nutrient deprivation. We found that eEF-2 kinase and autophagy were activated following starvation treatment in human breast cancer cells. Nutrient starvation also decreased mTOR activity, and reduced the incorporation rate of ^{35}S-methionine, indicating protein synthesis was inhibited. Silencing of eEF-2 kinase by RNAi relieved the inhibition of protein synthesis, and resulted in a greater reduction of cellular ATP. EEF-2 kinase-targeted RNAi also blunted autophagic response of the tumor cells. Inhibition of autophagy by knockdown of eEF-2 kinase or autophagy-related gene Beclin-1 impeded cell growth in serum/nutrient-deprived cultures and handicapped cell survival. These results indicate that in response to nutrient/growth factor deprivation breast cancer cells activates eEF-2 kinase and autophagy to decrease protein synthesis and regenerate ATP, and that inhibition of eEF-2 kinase renders cells continue to elongate peptide, deplete ATP, and impairs cancer cell survival under metabolic stress. Furthermore, we determined whether inhibition of autophagy sensitized breast cancer cells to growth factor antagonists. Synergistic effect on cell growth inhibition was observed from combination of a small molecule EGFR/ErbB-2 inhibitor with an autophagy inhibitor 3-methyladenine (combination index values at ED50 0.6279 and 0.7879, respectively). Inhibition of autophagy by knockdown of eEF-2 kinase or Beclin 1 sensitized breast cancer cells to the EGFR/ErbB-2 inhibitor and the mTOR inhibitor rapamycin. These results provide new evidence that activation of eEF-2 kinase and autophagy plays protective role for cancer cells under metabolic stress, and that targeting autophagic survival may represent a novel approach to sensitizing cancer cells to growth factor antagonists.

15. SUBJECT TERMS
Breast cancer; autophagy; elongation factor-2 kinase; growth factor antagonist

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	17	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

INTRODUCTION

We propose that breast cancer cells utilize autophagy ("self-digestion") to survive during times of nutrient and/or growth factor deprivation. For example, when cells lack an adequate blood supply, or treatment with drugs that block growth factors, a percentage of cells survive by slowing energy utilization and recycling their own proteins and damaged organelles. These "hibernating" cells can subsequently be revived when nutrients become available. Therefore, by inhibiting autophagy normal breast cells will be less able to survive malignant transformation and treatment with drugs that block growth factors.

Autophagy is a survival process first uncovered in yeast and is now known to be activated in response to nutrient deprivation in plants, worms, flies, mice, and man (1). In yeast, nutrient deprivation results in two outcomes: 1) self-digestion of cytoplasm and organelles and recycling of "spare parts" (e.g., amino acids) for energy utilization and; 2) the budding of an immortal spore. This response in unicellular organisms leads to self-preservation in times of famine. Not surprisingly, autophagy finds its counterpart in multicellular organisms, as high up the animal kingdom as man, where nutrient deprivation prompts cells to exit the cell cycle, shrink, digest long-lived proteins and damaged organelles, and recycle the components to maintain cellular energy. To support autophagy, cells must limit further use of energy, and have evolved a pathway to inhibit protein synthesis, the cell's major consumer of energy. Autophagy may thus result in cellular destruction or alternatively cellular "hibernation" until the supply of nutrients is restored.

Despite targeted therapies with selective estrogen receptor modulators (e.g., Tamoxifen), aromatase inhibitors (e.g. Arimidex), and antagonists of Her-2/neu (e.g., Herceptin), the response to these treatments are unacceptably limited. Much remains to be discovered about the mechanisms by which breast cancer cells escape the inhibitory effects of these medications. New and exciting data are emerging in model systems that suggest the possibility that breast cancer cells survive growth factor depletion/inhibition through autophagy. Furthermore, it is possible to inhibit this survival response by targeting one of several promising components of the autophagy pathway. We will test the hypothesis that breast cancer cells survive nutrient/growth factor deprivation through autophagy and that targeting a key component of the autophagy pathway will decrease breast cancer viability. One promising target is an enzyme, eEF-2 kinase, which is activated during autophagic survival to inhibit protein synthesis and conserve energy. Our rationale for targeting eEF-2 kinase is its: 1) overexpression in human breast cancer; 2) activation during autophagy; 3) ability to inhibit protein synthesis and conserve energy and; 3) unique structure, making this kinase amenable to selective inhibition. By blocking eEF-2 kinase in the face of nutrient/growth factor deprivation, protein elongation would continue and deplete critical supplies of energy resulting in cell death.

BODY

Task 1 To determine the importance of autophagy in the life and death of breast cancer cells.

We have been continuing our studies on the roles of eEF-2 kinase and autophagy in the response of cancer cells to environmental and metabolic stresss. We further verified that eEF-2 kinase and autophagy were activated following starvation treatment in human breast cancer cell lines. Nutrient starvation inhibited mTOR activity as evidenced by the decreases of phosphor-S6 kinase and phosphor-4EBP1 (Figure 1C), activated eEF-2 kinase as indicated by the increases of phospho-EF-2 (Figure 1A and B), and inhibited protein synthesis (reduced the incorporation rate of ^{35}S-methionine) (Figure 1D). Nutrient deprivation also caused reduction of cellular ATP content (Figure 2A) and activation of AMP kinase (Figure 2B), and induced autophagic response, as measured by LC3-II turnover (Figure 3A) and GFP-LC3 cleavage (Figure 3B) assays. Silencing of eEF-2 kinase by RNA interference (RNAi) relieved the inhibition of protein synthesis (Figure 4B), and resulted in a greater reduction of cellular ATP (Figure 5). eEF-2 kinase-targeted RNAi also blunted autophagic response of the tumor cells (Figure 4A). Inhibition of autophagy by knockdown of eEF-2 kinase or autophagy-related gene Beclin-1 impeded cell growth in serum/nutrient-deprived cultures and handicapped cell survival (Figure 6). These results indicate that in response to nutrient/growth factor deprivation breast cancer cells activates eEF-2 kinase and autophagy to decrease protein synthesis and regenerate ATP, and that inhibition of eEF-2 kinase renders cells continue to elongate peptide, deplete ATP, and impairs cancer cell survival under metabolic stress. These results are shown in the Appendix. These observations provide further evidence that eEF-2 kinase is activated in response to metabolic stress, and that inhibiting eEF-2 kinase may overcome the cellular attempts to survive via autophagic regeneration of ATP.

Task 2 To determine the role of autophagy in the sensitivity of breast cancer to treatment.

During the last grant we determined the effect of autophagy on the sensitivity of breast cancer cells to small molecule antagonists of growth factor receptor such gefitinib and lapatinib. We showed that treatment of human breast cancer cells, MDA-MB-468, with gefitinib (2.5 μM and 5.0 μM) and lapatinib (5.0 μM and 10 μM) activated autophagy (Figure 7), as examined by autophagic flux using GFP-LC3 cleavage assay. We also demonstrated that inhibition of autophagy by autophagy inhibitor, 3-methyladenine (3-MA), enhanced the sensitivity of breast cancer cell lines SKBR3 and MDA-MB-468 to a small molecule EGFR/ErbB-2 inhibitor (combination index values at ED50 0.6279 and 0.7879, respectively) (Table 1). Suppression of autophagy by knockdown of eEF-2 kinase expression or autophagy gene Beclin 1 expression also sensitized SKBR3 and MDAMB468 cells to the growth – inhibitory effects of EGFR/ErbB-2 inhibitor and the mTOR inhibitor rapamycin (Table 2). Furthermore, we showed that silencing of expression of eEF-2 kinas and beclin 1 potentiates the growth – inhibitory effects of lapatinib and gefitinib on MDA-MB-468 and SKBR3 cells (Table 3). The results of these studies are shown in the Appendix. These results provide new evidence that activation of eEF-2 kinase and autophagy plays cyto-protective role for cancer cells under

metabolic stress, and that targeting autophagic survival may represent a novel approach to sensitizing cancer cells to growth factor antagonists.

KEY RESEARCH ACCOMPLISHMENTS

- We have validated that eEF2 kinase and autophagy are activated in response to nutrient/growth factor deprivation in breast cancer cells. Inhibition of eEF2 kinase by RNAi knockdown inhibited autophagy activity.
- We observed that following nutrient/growth factor deprivation, inhibition of eEF2 kinase by RNAi knockdown relieves the inhibition of protein synthesis, and results in a greater and more rapid reduction of cellular ATP in breast cancer cells.
- We demonstrated that inhibition of autophagy by RNAi knockdown of eEF2 kinase or autophagy-related genes handicapps cell survival in serum-deprived cultures.
- We showed that there is a synergistic effect from combination of growth factor antagonist EGFR/ErbB-2 inhibitor with autophagy inhibitor 3-MA in SKBR3 and MDAMB468 cells.
- We also found that inhibition of BECN1 or EF2K by RNAi knockdown sensitizes MDAMB468 and SKBR3 cells to the treatment of EGFR/ErbB-2 inhibitor lapatinib and EGFR inhibitor gefitinib.

REPORTABLE OUTCOMES

Manuscript
None

Abstracts

Li HM, Hait WN, Yang JM: Targeting autophagic survival pathway sensitizes human breast cancer cells to growth factor antagonists. Proc Amer Assoc Cancer Res 49: 4935, 2008.

Degree obtained that are supported by this award
None

CONCLUSIONS

Targeting eEF-2 kinase – regulated autophagic survival pathway may stand for a new strategy of sensitizing human breast cancer cells to therapy with growth factor antagonists.

REFERENCES

1. Shintani, T. and Klionsky, D. J. Autophagy in health and disease: a double-edged sword. Science, *306:* 990-995, 2004.

APPENDIX

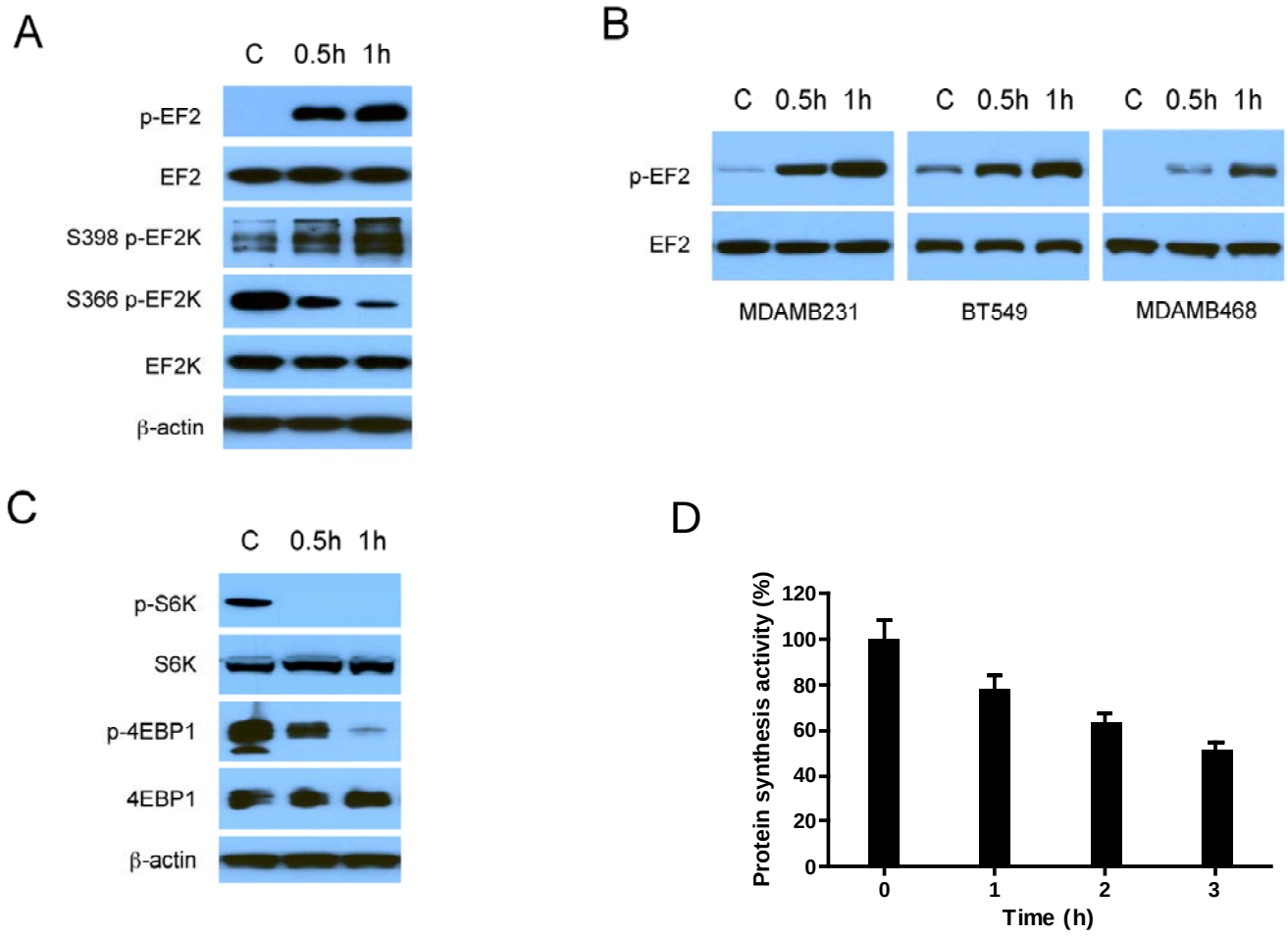


Figure 1. Nutrient starvation inhibits mTOR/S6 kinase, activates eEF-2 kinase, and decreases protein synthesis activity in human breast cancer cells.

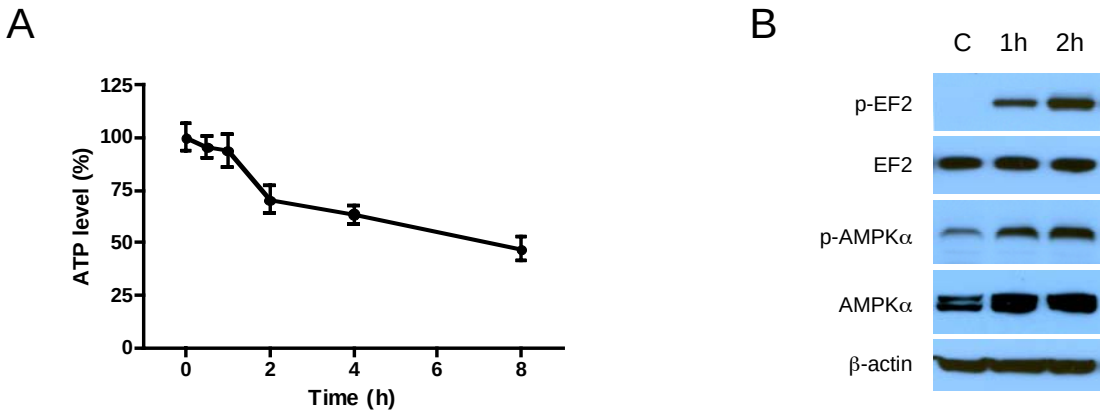


Figure 2. Nutrient starvation decreases cellular ATP level and activates AMP kinase in human breast cancer cells.

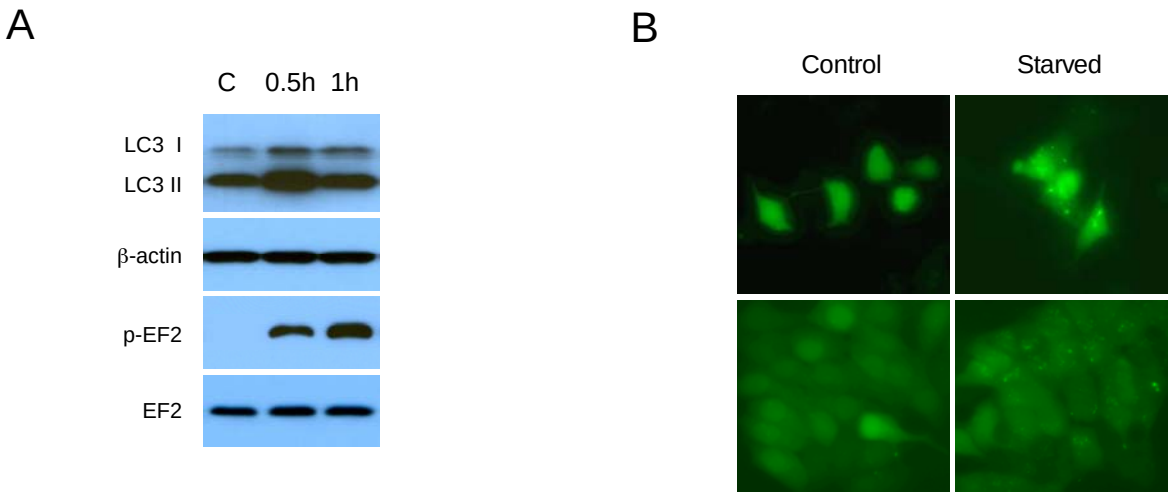


Figure 3. Nutrient starvation activates autophagy in MCF-7 human breast cancer cells.

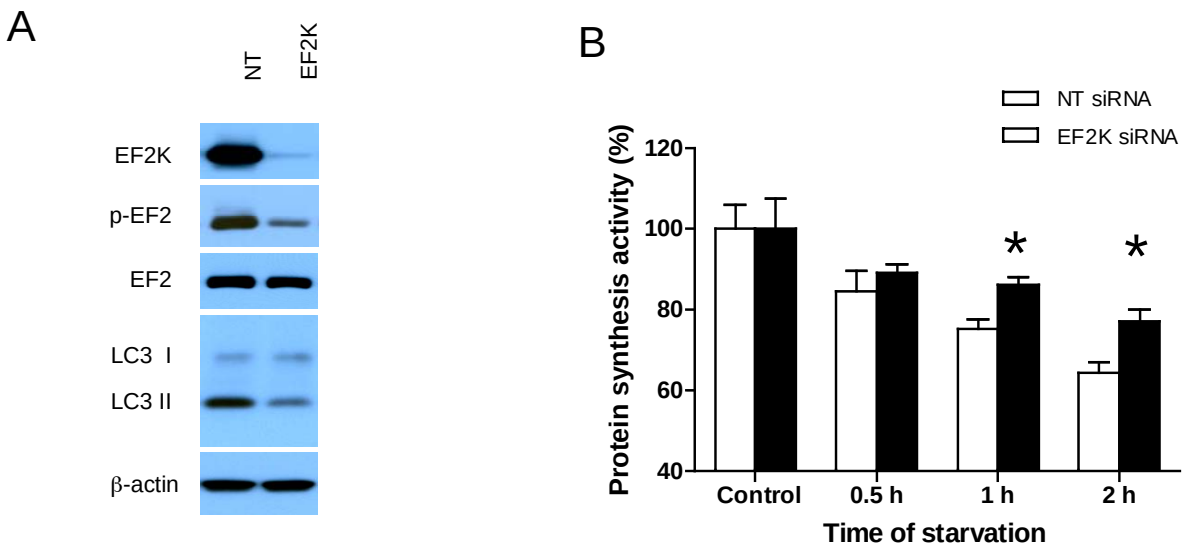


Figure 4. Knockdown of eEF-2 kinase expression blunts autophagic response and relieves the Inhibition of protein synthesis following nutrient deprivation.

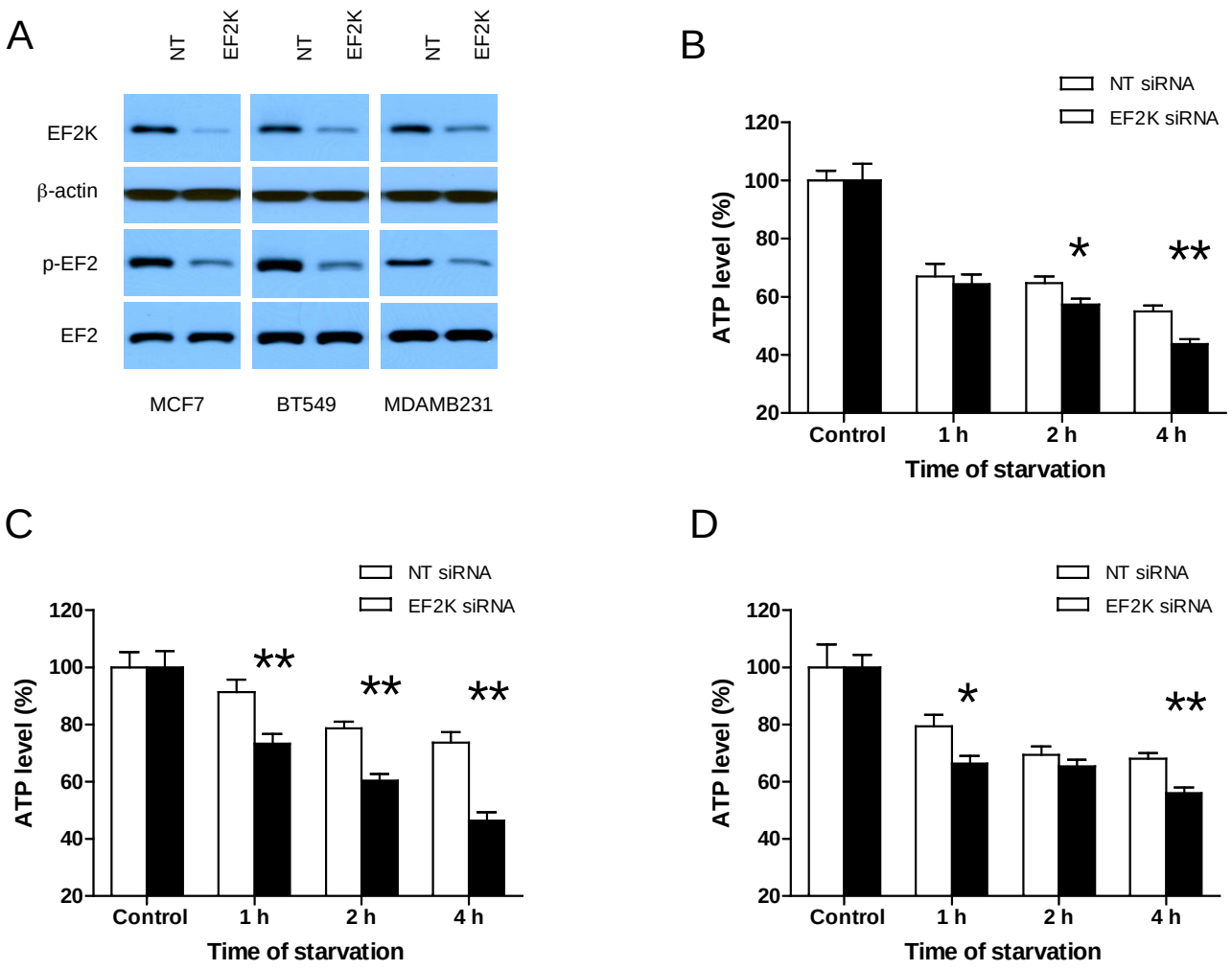


Figure 5. Knockdown of eEF-2 kinase expression hastens the the decrease of cellular ATP level following nutrient deprivation

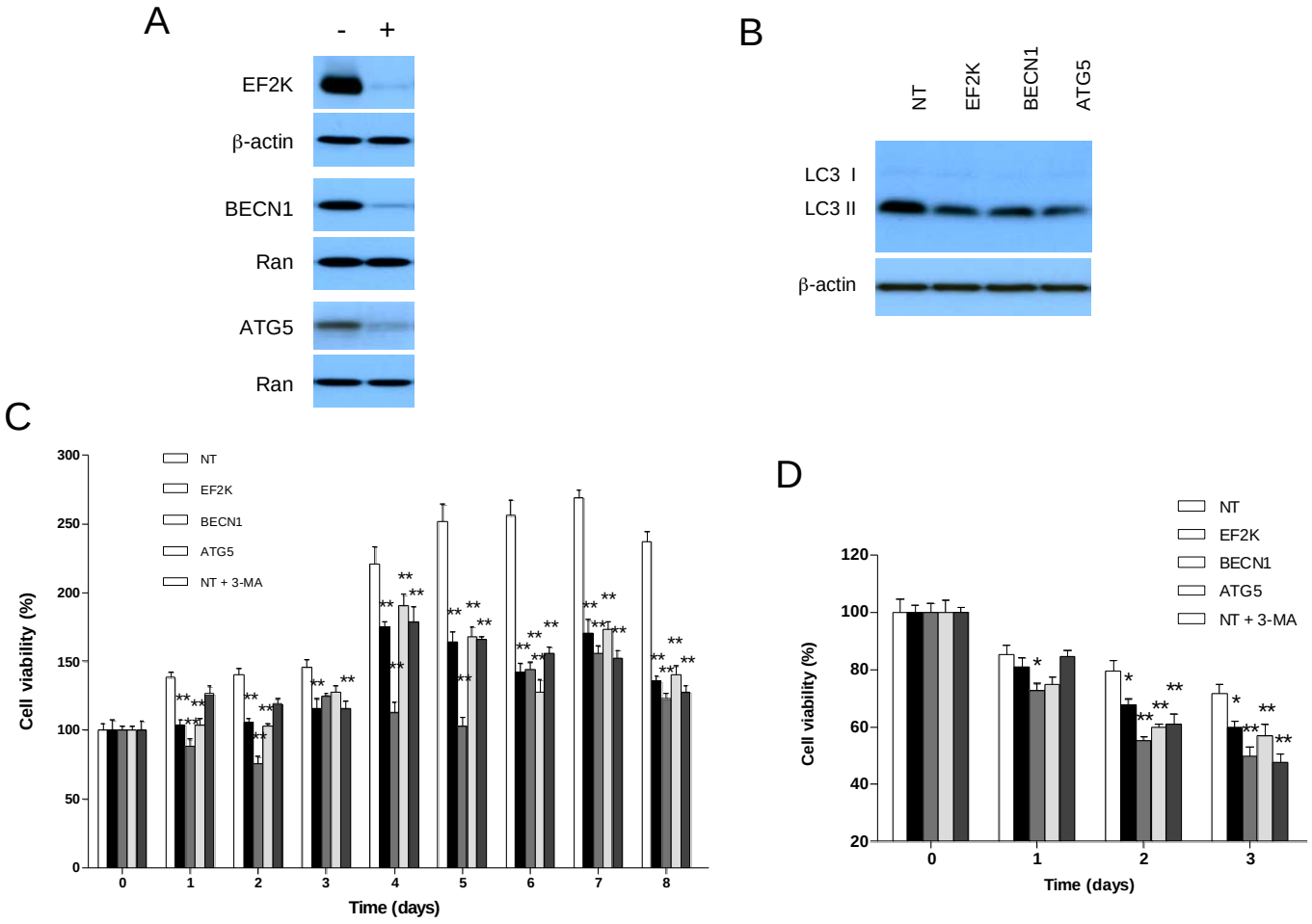


Figure 6. Silencing of eEF-2 kinase or autophagy genes beclin1 and ATG5 impairs survival of human breast cancer cells under metabolic stress.

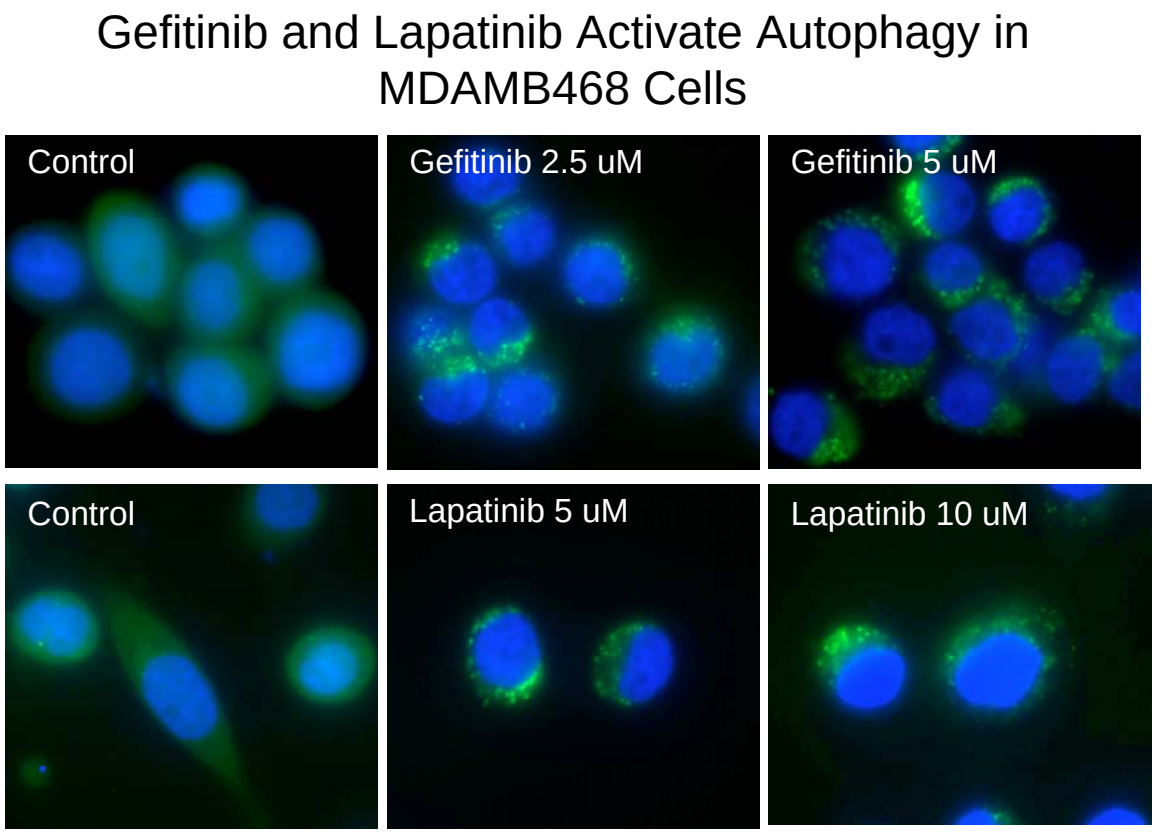


Figure 7. Treatment of human breast cancer cells with growth factor antagonists induces autophagic response.

Synergy Detection: combination of EGFR/ErbB2 inhibitior and 3-MA

Cell line	ED10	ED25	ED50	ED75	ED90
MDAMB468	0.5396	0.4786	0.6297	0.9253	1.3903
SKBR3	1.5949	1.0169	0.7975	0.7017	0.6492

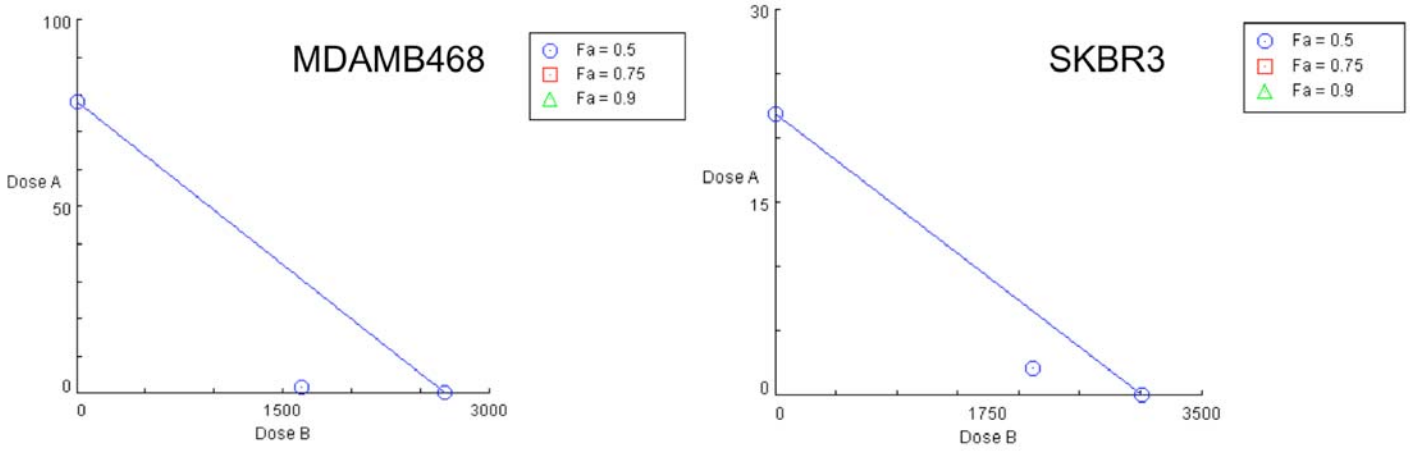


Table 1. Inhibition of autophagy by 3-MA enhances the sensitivity of breast cancer cells to the growth-inhibitory effect of a small molecule inhibitor of EGFR/ErbB-2. Numbers shown are combination indices.

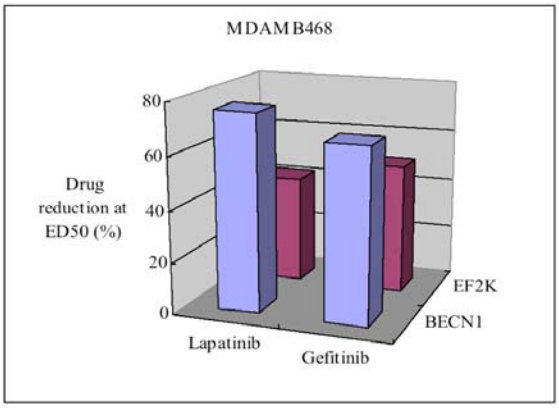
Drug Sensitivity: EF2K knockdown in SKBR3

Drug	SiRNA	ED25	ED50	ED75
EEi (uM)	NT	2.9113	9.5804	31.528
	EF2K	2.2677	8.2306	29.873
	Reduction	-21.1%	-14.1%	-5.2%
Rap (nM)	NT	0.3548	13.243	494.36
	EF2K	0.1265	6.2066	304.54
	Reduction	-64.3%	-53.1%	-38.4%

Table 2. Silencing of eEF-2 kinase expression increases the sensitivity of breast cancer cells to the growth-inhibitory effects of rampamycin and a small molecule inhibitor of EGFR/ErbB-2.

Drug Reduction at ED50: Effect of BECN1 and EF2K knockdown

Drug	SiRNA	MDAMB468	SKBR3
Lapatinib (uM)	NT	16.912	1.723
	BECN1	4.073	0.692
	NT	18.624	2.356
	EF2K	10.791	1.134
Gefitinib (uM)	NT	3.893	4.137
	BECN1	1.284	2.433
	NT	3.312	3.912
	EF2K	1.654	3.314



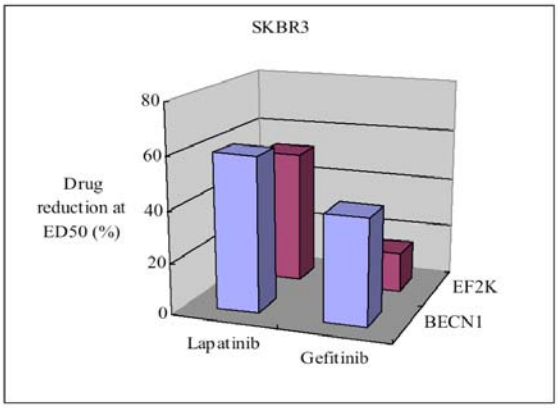


Table 3. Silencing of expression of eEF-2 kinase or autophagy gene, beclin1, increases the sensitivity of breast cancer cells to the growth factor antagonists, lapatinib and gefitinib.